The Measurement of Theophylline Metabolism in Hepatic Microsomes Using High-Performance Liquid Chromatography

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Abstract □ A method is described to measure the *in vitro* metabolism of theophylline by liver microsomes. The formation of 1-methyluric acid and 1,3-dimethyluric acid in incubation mixtures was determined by high-performance liquid chromatography. The formation of both metabolites was linear with time and the formation of 1-methyluric acid was blocked by allopurinol. This method will be useful in assessing potential drug interactions involving theophylline.

Keyphrases □ Theophylline—metabolism in hepatic microsomes, high-performance liquid chromatographic measurement □ Microsomes, hepatic—measurement of theophylline metabolism, high-performance liquid chromatography □ High-performance liquid chromatography—measurement of theophylline metabolism in hepatic microsomes □ Metabolism—theophylline in hepatic microsomes, high-performance liquid chromatography

Theophylline (1,3-dimethylxanthine) is a widely used antiasthmatic drug which is metabolized by hepatic microsomal enzymes (1). The use of theophylline can be problematic because of the narrow therapeutic range of serum concentrations and because of the large number of factors which can alter its metabolism and ultimate elimination (2). An *in vitro* system to study theophylline metabolism would be a useful technique to examine and define many of the factors which alter the elimination of this drug. Such a method for the study of radiolabeled theophylline has been described previously (3), but this technique was successful only with liver slices or with microsomes from animals previously induced with 3-methylcholanthrene. The present report discusses an improved method for the determination of theophylline metabolism in normal liver microsomes using high-performance liquid chromatography to quantitate the metabolites.

EXPERIMENTAL

Reagents and Chemicals—Theophylline[1], theobromine[1], 1,3-dimethyluric acid[1], 8-chlorotheophylline[1], and 1-methyluric acid[2] were used as received.

Chromatography—A liquid chromatograph[3] with reciprocating dual-piston constant flow rate pump and rotary valve injector[4] was equipped with a 20-μl loop, HPLC reversed-phase column[5], variable wavelength UV detector[6], and recorder[7]. The mobile phase contained 0.01 *M* sodium acetate–1% acetic acid–methanol (64:21:15, v/v/v). The solvent was degassed before use. Mobile phase flow rate was 0.4 ml/min.

Calibration Curves—Standard solutions which contained 0.10–10 μg/ml of 1-methyluric acid or 0.25–25 μg/ml of 1,3-dimethyluric acid and 100 μg/ml of 8-chlorotheophylline or 20 μg/ml of theobromine as internal standard were extracted and chromatographed in an identical manner to the incubation mixtures. Standard curves were linear throughout the range utilized.

Microsomal Biotransformation—Microsomes (3 mg of protein) were incubated in media (3) containing 50 m*M* tromethamine hydrochloride pH 7.5, 5 m*M* $MgCl_2$, 10 m*M* D-glucose-6-phosphate, 1 m*M* triphosphopyridine nucleotide, 10 U glucose-6-phosphate dehydrogenase, and 0.25 m*M* theophylline in a total volume of 3 ml. Incubations were carried out at 37° in a metabolic shaking water bath[8]. After 2 hr of incubation the mixture was placed on ice and 100 μg/ml of 8-chlorotheophylline was added. The incubation mixture was then extracted with 3 ml of chloroform–isopropanol (90:10, v/v) for 30 min to remove unmetabolized theophylline. The aqueous layer was dried in a stream of air and then reconstituted in a 100-μl HPLC mobile phase for chromatography using 295 nm for detection.

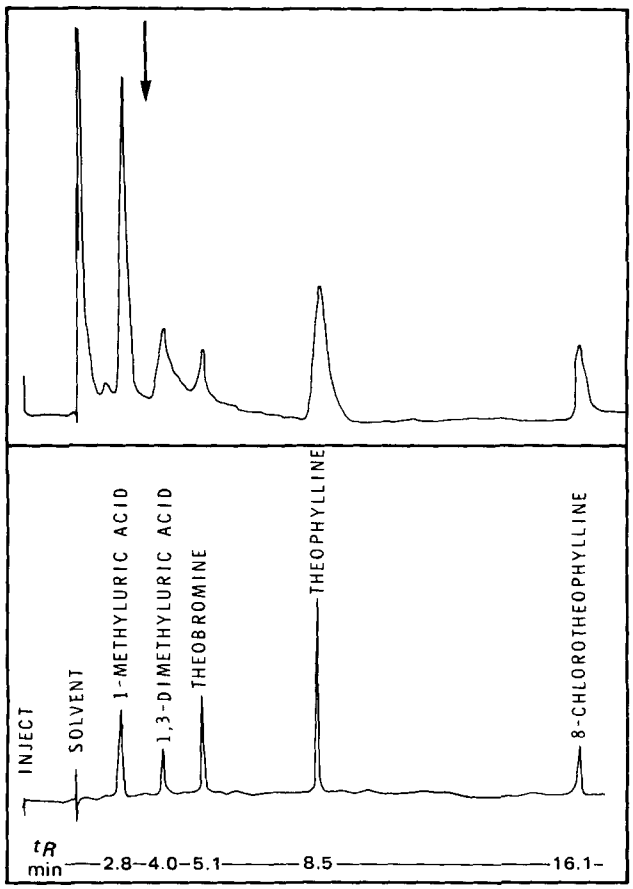

Figure 1—*HPLC for theophylline metabolites and internal standards. HPLC of extracted liver microsomes incubated with theophylline (upper). HPLC of 1-methyluric acid, 1,3-dimethyluric acid, theobromine, theophylline, and 8-chlorotheophylline standards (lower).*

[1] Sigma Chemical Co. St. Louis, Mo.
[2] Gift from Dr. J. Williams, University of Florida.
[3] Spectrophysics SP3500B.
[4] Rheodyne Model 7120.
[5] RP-8, 10 μm Brownlee Labs.
[6] Shoeffel UV variable wavelength detector.
[7] Fisher Recordall Series 5000.
[8] Dubnoff.

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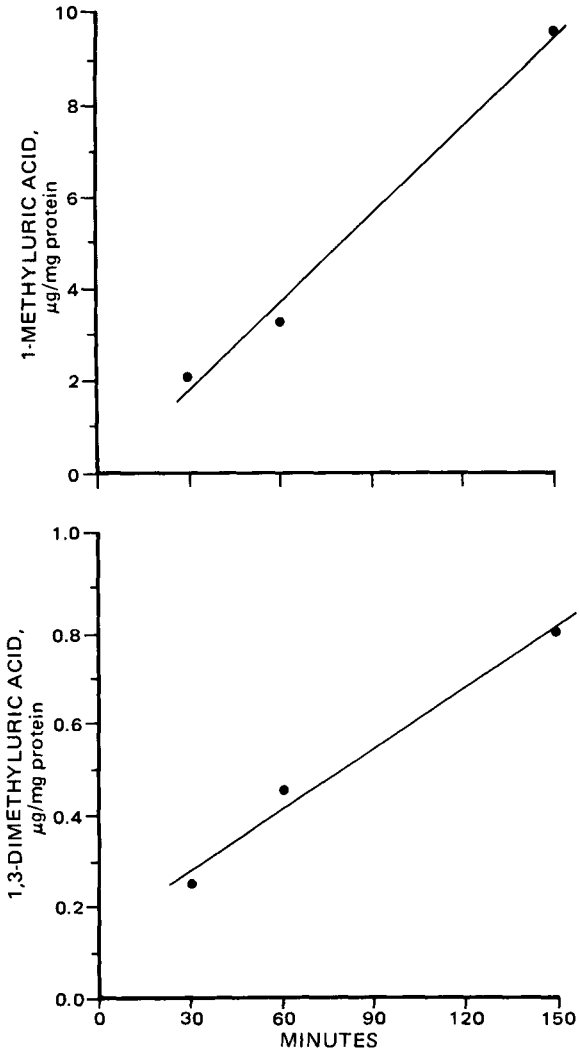

Figure 2—*Effect of increasing incubation time on production of 1-methyluric acid and 1,3-dimethyluric acid. The production of 1-methyluric acid (upper) and 1,3-dimethyluric acid (lower) from theophylline in microsomes prepared from rabbit liver were determined at 30, 60, and 150 min of incubation in a metabolic shaking water bath*[8].

Microsomes—Hepatic microsomes were prepared from rabbits using ultracentrifugation (4). Microsomal protein was determined by the method of Lowry using bovine serum albumin as a standard (5).

RESULTS AND DISCUSSION

A chromatogram of a hepatic microsomal incubation mixture demonstrated a clear separation of peaks for 1-methyluric acid, 1,3-dimethyluric acid, theophylline, theobromine, and 8-chlorotheophylline and is compared to a chromatogram of standards in Fig. 1. The production of 1-methyluric acid and 1,3-dimethyluric acid from theophylline by hepatic microsomes was determined to be linear for at least 150 min of incubation time (Fig. 2). A 2-hr incubation period was determined to give adequate amounts of metabolite for most experimental purposes. The amount of metabolites formed *in vitro* by rabbit liver microsomal enzyme metabolism of theophylline was 0.17 ± 0.04 μg of 1,3-dimethyluric acid/mg of protein/hr and 1.08 ± 0.58 μg of 1-methyluric acid/mg of protein/hr. Each value represents the mean ± standard error of microsomal preparations from four individual animals. The production of 3-methylxanthine from theophylline could not be detected in these incubation mixtures.

Table I—*In Vitro* Effect of Allopurinol[a] on *In Vitro* Theophylline Metabolism in Rabbits

	Control	Allopurinol[a]
1,3-Dimethyluric acid, μg/mg of protein/hr	0.04[b]	0.04
1-Methyluric acid, μg/ml of protein/hr	2.66	Not detectable

[a] 10^{-4} *M* allopurinol was added to microsomal incubation mixture. [b] Each value represents an individual incubation mixture using microsomes from the same control animal.

Allopurinol has been reported to decrease production of 1-methyluric acid from 1-methylxanthine (6, 7) by inhibiting xanthine oxidase. In this preparation, the addition of allopurinol (10^{-4} *M*) to the incubation mixture blocked production of 1-methyluric acid (minimum detectable limit 0.015 μg/mg of protein/hr) while not affecting production of 1,3-dimethyluric acid (Table I). This confirms that in this incubation system, 1-methyluric acid was being produced along the predicted metabolite route.

This method for the measurement of the metabolism of theophylline using an *in vitro* microsomal preparation does not require previous induction of hepatic microsomes and provides a rate of formation of specific major metabolites of theophylline. Use of this method can determine whether a change in theophylline elimination is due to specific changes in metabolite production and will also be useful in the investigation, assessment, and prediction of drug interactions involving alterations of theophylline metabolism.

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